IMMUNOLOGICAL UNRESPONSIVENESS IN ALLERGIC CONTACT DERMATITIS TO DINITROCHLOROBENZENE IN GUINEA PIGS*

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In previous experiments (1, 2) dealing with allergic contact dermatitis of the guinea pig to 2:4-dinitro-1-chlorobenzene (DNCB), a partial or total, but short-lived inhibition of the hypersensitivity state could be obtained by injecting intravenously DNCB or the immunologically related 2:4-dinitrobenzene sulfonic acid sodium salt ($DNBSO_3$) in already sensitized animals.

In the present experiments, a permanent state of unresponsiveness was obtained by giving $DNBSO_3$ intravenously *before* sensitization. So pretreated animals did not become sensitized and remained unresponsive after repeated epicutaneous applications of DNCB. These results confirm the early work of Sulzberger (23), as well as that that of Chase (3, 4) who showed that the administration of the allergen prior to the sensitizing application may induce a total or partial immunological unresponsiveness in adult animals, and not only in newborn animals usually considered for immunological tolerance (5).

MATERIALS AND METHODS

2:4-dinitrobenzenesulfonic acid sodium salt ($DNBSO_3$) was obtained from Eastman Kodak, Rochester, N. Y.; 2:4-dinitro-1-chlorobenzene (DNCB), reagent grade, from Merck, Darmstadt; and α-2-chloro-9 (3-dimethylaminopropylidene) thioxanthene hydrochloride, Chlorprothixene hydrochloride (CPT) from Hoffmann-La Roche, Inc., Basle. White semi-inbred guinea pigs of both sexes weighing 400–500 g were used throughout.

The general disposition of our experiments consisted in a) pretreating the animals with $DNBSO_3$, b) sensitizing them with DNCB and/or CPT and c) testing them later with these compounds in order to determine the effect of the pretreatment or their capacity to react.

Pre-treatment

$DNBSO_3$ was administered as one single i.v. injection in a vein of the hind leg in doses of 750, 500, 250 and 125 mg/kg to different groups of 6–8 guinea pigs 28, 14, 7, 3, 2 and 1 day before and 1 day after the sensitizing contact with DNCB. $DNBSO_3$ was dissolved in bi-distilled water or saline and, according to the dose, concentrations of 25, 12, 5 and 6.25% were used. The volumes injected varied from 0.8 to 1.5 ml.

Sensitization Technic

a) DNCB: 0.002 ml of a 50% solution of DNCB in acetone was applied with a micro-pipette on the epilated skin of the nuchal region on an area of 2–5 mm diameter. This technic published elsewhere (6) induces sensitization in 100% of the animals used.

b) CPT: Intradermal injection of 0.1 ml of a 20% aqueous solution was given on three alternate days.

c) In order to determine the specificity of the phenomena investigated, one group of 16 guinea pigs was pretreated with $DNBSO_3$, then sensitized simultaneously to DNCB and CPT and tested weekly with both substances.

Skin Testing

a) DNCB: 14 days after sensitization and then at weekly intervals, the animals were tested with 3 different concentrations of DNCB in acetone by applying 0.025 ml of a 0.9, 0.5 and 0.3 per mille solution on a circular area of 2 cm^2 of the skin of the flank.

b) CPT: Epicutaneous application of 0.025 ml of aqueous solutions of 10, 3 and 1 per cent.

The tests were read 24 hours later and evaluated as follows: 0.5: some red spots in the test area; 1: slight reddening; 2: marked reddening and swelling; 3: marked reddening and marked swelling.

The sum of the readings obtained with three different test concentrations gives some numerical estimate of the hypersensitivity level of the animal tested and of the average sensitivity of a group as published elsewhere (6).

Controls

a) 32 guinea pigs not pretreated but similarly sensitized and tested with DNCB (positive controls).

b) 18 animals neither pretreated nor sensitized but tested with DNCB at the same intervals as the experimental groups (negative controls).

EXPERIMENTS AND RESULTS (TABLE 1)

Our experiments were performed in order to determine: the influence of intravenously administered $DNBSO_3$ (pretreatment) on the capacity of immunologic response to subsequent epicutaneous sensitization with DNCB, the influence of the dose and the time of injection of $DNBSO_3$ on the occurrence of unresponsiveness, the duration

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TABLE I

Unresponsiveness to DNCB contact in guinea pigs pretreated by one intravenous injection of $DNBSO_3$

Experiment	$DNBSO_3$ i.v.		− = Unresponsive + = Responsive	Results* of Skin Tests With DNCB According to Weeks After Sensitizing Application of DNCB†							
				2		3‡		16		38	
	Days before primary contact	mg/kg		Number of animals	Intensity§	Number of animals	Intensity	Number of animals	Intensity	Number of animals	Intensity
1	28	500	−	4	0.5	4	0.8				
			+	4		4					
2	14	750	−	4	0.5	3	1.0	2	1.5	2	0.5
			+	2		3		1		1	
3	14	500	−	10	0.7	7	1.1	2	1.1	2	0.5
			+	5		6		3		3	
4	14	250	−	2	1.1	2	1.4	0	1.1	0	0.5
			+	5		5		5		4	
5	14	125	−	0	1.0	0	1.7	0	1.3	0	0.6
			+	8		6		6		5	
6	7	750	−	2	0.8	2	0.6				
			+	4		4					
7	7	500	−	5	0.7	5	1.0	1	1.1	1	1.0
			+	10		10		4		4	
8	3	750	−	2	0.5	2	0.6				
			+	5		5					
9	3	500	−	0	0.8	0	1.1	0	1.0	0	0.8
			+	8		7		3		2	
10	2	500	−	0	1.6	0	1.5	0	0.8	0	0.5
			+	7		7		3		3	
11	1	500	−	0	0.9	0	1.2	0	1.3	0	0.7
			+	8		8		6		6	
12	1 day after primary contact	500	−	0	1.6	0	1.7	0	1.5	0	0.8
			+	8		8		7		6	
13	Positive controls‖		−	0	2.7	0	3.4	0	2.4	0	2.6
			+	32		31		18		12	
14	Negative controls¶		−	18	0	9	0.5	0	2.5		
			+	0		9		18			

* Epicutaneous tests with DNCB were carried out weekly for 24 weeks with 0.9, 0.5 and 0.3% solutions of DNCB in acetone. Only the figures for weeks 2, 3, 14, 16, 20 and 24 are mentioned.

† The animals were sensitized by applying 0.002 ml of a 50% solution of DNCB in acetone epicutaneously.

‡ Second sensitizing application 14 weeks after first.

§ Intensity expressed as mean of the three test reactions.

‖ No pretreatment but sensitizing application and skin testing.

¶ No pretreatment, no sensitizing application, only skin testing.

For further details see chapter II.

of the diminished or abolished responsiveness and the effect of repeated epicutaneous application and testing on unresponsiveness.

Design of the experiments and the results are summarized in Table 1 and may be analyzed as follows:

a) Number of animals completely unresponsive compared to total number of animals pretreated and sensitized.

b) Sensitivity level of the partially responsive animals.

c) Duration of these effects.

It may be concluded that pretreatment with $DNBSO_3$ induces in a high proportion of the animals a complete unresponsiveness to a subsequent sensitizing contact with DNCB (experiments 1, 2, 3, 4, 7 and 8). This effect persists for 38 weeks despite the weekly testing and a second sensitizing application performed in the surviving animals (experiments 2, 3 and 7).

The unresponsiveness seems to be proportional to the dose of $DNBSO_3$ administered (experiments 2 and 3 versus 4 and 5) as no effect is obtained when less than 250 mg/kg are given. The proportion of unresponsive animals could not be enlarged by administering higher doses of $DNBSO_3$ when injected 14 or 7 days before sensitization (experiments 3 versus 2 and 7 versus 6). However, when 750 mg/kg $DNBSO_3$ was given 3 days before the sensitizing contact (experiment 9 versus 8), high doses were definitely more effective.

The unresponsiveness is further related to the time of injection of the hapten. No effect is observed with 500 mg/kg when less than 7 days elapse between pretreatment and sensitization (experiments 3 versus 7, 9–12). However, when higher doses of $DNBSO_3$ are given as late as 3 days before the sensitizing application (experiment 8), unresponsiveness is still obtained.

Complete unresponsiveness in a similar proportion of animals is obtained when pretreatment is performed 28, as well as 14, days before sensitization (experiments 3 and 2 versus 1).

In those pretreated animals which *responded* to sensitization, the acquired degree of sensitivity was distinctly lower than that of the positive controls. In fact, their sensitivity level oscillates around 0.5 to 1.7, whereas the controls showed values of 2.4 to 3.4. This partial responsiveness occurred without apparent correlation to the dose and the time of injection of $DNBSO_3$; it persisted for 38 weeks; and could not be raised either by repeated testing or by a second sensitizing application.

In the non-pretreated but similarly sensitized group (positive controls, experiment 13), all the animals did respond, their sensitivity level reaching the usual high value which remained constant until the end of the experiment.

In the non-pretreated and not sensitized group (negative controls, experiment 14), the sensitizing effect of repeated testing with "primarily non-irritating" concentrations of DNCB is shown. All these animals became positive at the time of the fourth test and their sensitivity level was comparable to the level of positive controls at the time of the fifth test.

Specificity of Unresponsiveness (Table 2)

In order to determine whether unresponsiveness due to pretreatment is a specific phenome-

TABLE 2

Specificity of the unresponsiveness in guinea pigs pretreated by one intravenous injection of $DNBSO_3$ and double-sensitized 14 days later by epicutaneous application of DNCB and/or intradermal injections of CPT

Experiments	Number of Animals	Pretreatment	Sensitization	Skin-testing with	Skin Tests at Weeks					
					2			6		
					Number of animals		Intensity	Number of animals		Intensity
					Unresponsive.	Responsive.		Unresponsive.	Responsive.	
1	16	500 mg/kg $DNBSO_3$ i.v. 14 days before sensitization	DNCB e.c. and CPT i.d.	DNCB	10	6	0.7	6	7	1.2
				CPT	0	16	1.87	0	13	2.27
2	8	—	DNCB e.c. and CPT i.d.	DNCB	0	8	2.63	0	7	3.21
				CPT	0	8	2.19	0	7	2.63
3	7	—	DNCB e.c.	DNCB	0	7	2.36	0	6	3.0
4	8	—	CPT i.d.	CPT	0	8	2.07	0	8	2.62
5	8	—	—	DNCB	8	0	0	0	7	2.2
				CPT	8	0	0	1	6	0.8

non, one group of 16 animals (experiment 1) where pretreated with $DNBSO_3$ and 14 days later sensitized concomitantly to two immunologically unrelated compounds (DNCB and CPT). The following necessary positive and negative controls were done:

no pretreatment but double sensitizing and testing (experiment 2)

no pretreatment but sensitizing and testing either with DNCB or CPT (experiments 3–4)

no pretreatment, no sensitization, but testing with both substances (experiment 5).

Results are summarized on Table 2. Complete unresponsiveness to DNCB was obtained in a high proportion of the double sensitized animals, those responding acquired only a low level of sensitivity. The concomitant sensitization to CPT was not influenced by the pretreatment and the same sensitivity level was acquired as in the controls. All non-pretreated and non-sensitized animals became positive after performing 4 tests, *i.e.* after 5 weeks.

SEROLOGICAL STUDIES

Material and Methods

Precipitating anti-DNP antibody was investigated by ring test using various DNP-protein conjugates as antigens. Among these, DNP-bovine gamma globulin (DNP-BγG), DNP-human serum albumin (DNP-HSA) and DNP-guinea pig serum (DNP-GPS) were prepared by the method of Eisen (7), dialyzed extensively and passed through IRA-400 columns (8). The extent of substitution of protein carriers by DNP haptenic groups was determined spectrophotometrically (8). When feasible, quantitative precipitin analysis was carried out (9).

Hemagglutination was performed by using tanned sheep red blood cells incubated with DNP-BγG or DNP-HSA at a concentration of 1–3 mg protein/ml (10). The sensitivity of this assay, as determined with guinea pig antisera of known anti-DNP precipitating antibody content, was found not to exceed 5 μg Ab protein/ml.

Passive cutaneous anaphylaxis was performed according to Ovary (11), using 5 mg DNP-BγG or DNP-HSA as antigen. A latency time of 6 hours between the intradermal injection of the sera and

TABLE 3

Circulating anti-DNP antibodies and immediate-type hypersensitivity in guinea pigs after injection of DNP-BγG, DNCB, $DNBSO_3$ and/or epicutaneous application of DNCB

Results: positive animals/animals tested

Immunization procedure	Time of test: day	Precipitin analysis	Hemagglutination	Passive cutaneous anaphylaxis	Anaphylaxis		Contact reaction to DNCB
					Death	Symptoms	
DNP_{20}-BγG in Freund's adjuvant (62-235 mμM DNP/guinea pig) intradermal	14	16++/16*	16++/16†	16++/16§	12/12		2±/12
DNCB in Freund's adjuvant (235 mμM/guinea pig) intradermal	14	0/4	4+/4‡	4+/4‖	8/8		8++/8
DNCB epicutaneous (5 μM/guinea pig)	14	0/16	0/16	0/8	0/16	13+/16	16++/16
$DNBSO_3$ intravenous (2000 μM/guinea pig)	14	0/10	0/10	0/10	0/8	8±/8¶	0/14
	28	0/6	0/6	0/6	n.d.	n.d.	0/8
$DNBSO_3$ intravenous (2000 μM/guinea pig) on day 0, 5μM DNCB epicutaneous on day 14	28	0/13	0/13	0/13	0/2	0/2	5±/15

* Quantitative analysis on pool: 2.77 mg anti-DNP antibody protein/ml.

† Titers with DNP_9-HSA incubated, tanned sheep red blood cells: 1/2560–1/10240.

‡ Titers with DNP_9-HSA incubated, tanned sheep red blood cells: 1/2560–1/10240.

§ With DNP_9-HSA (5mg) and 1% Evans Blue, positive until 0.3–0.5 μg Ab protein/0.1 ml.

‖ Estimated from dilution and reaction sizes: 20–40 μg Ab/ml.

¶ Questionable symptoms of anaphylactic shock.

n.d. Not done.

the intravenous injection of antigen was found as satisfactory as the latency time of 17 hours, which has been sometimes recommended (12, 13). The sensitivity of this assay was found to be 0.3–0.5 μg Ab protein/0.1 ml and is in agreement with data published (13, 14).

Active anaphylaxis was induced by intravenous injection of DNP-GγG or DNP-GPS conjugate at doses of 0.8–8 μM DNP groups.

Serological Results (Table 3)

The following groups of differently sensitized animals were investigated serologically:

1) Animals sensitized by four *intradermal* injections of DNCB (0.235 μM) *in Freund's adjuvant* in each foot-pad did exhibit a high level of contact hypersensitivity and an intravenous injection of DNP-protein conjugates elicited regularly a severe or lethal anaphylactic shock. Circulating anti-DNP antibodies were demonstrated by passive cutaneous anaphylaxis (PCA) and hemagglutination (Table 3).

2) Animals sensitized by one single *epicutaneous* application of 0.002 ml of 50% DNCB *in acetone* showed also a high level of contact hypersensitivity and an intravenous injection of DNP-protein conjugates elicited a characteristic but not lethal anaphylactic shock (Table 3). The sera of these animals were investigated 14 days after sensitization for anti-DNP antibodies by PCA and hemagglutination. No detectable anti-DNP antibodies were found (Table 3).

3) Finally, animals pretreated *intravenously* with $DNBSO_3$ did not exhibit contact hypersensitivity and, when injected with DNP-protein conjugates 15 days after pretreatment, showed only questionable symptoms of anaphylactic shock. When sera of these animals were investigated for anti-DNP antibodies 4 and 28 days after the intravenous injection of $DNBSO_3$, as well as before and after the sensitizing epicutaneous application of DNCB, in no instance were anti-DNP antibodies detectable (Table 3).

It may be concluded from these serologic investigations that no anti-DNP antibodies are detectable by the technic currently used in animals sensitized epicutaneously with DNCB (quoted under 2) and in animals receiving $DNBSO_3$ intravenously alone and subsequently DNCB by epicutaneous application (quoted under 3). As these animals show weak or questionable anaphylactic reactivity they must have circulating anti-DNP antibodies at levels lower than detectable by the technics used (0.9 Ab N μg/ml).

DISCUSSION

If one compares our results obtained with intravenous injection of $DNBSO_3$ with those reported previously by Chase (3), with the feeding method, one will see that they are very similar.

The unresponsiveness obtained in our animals is specific, durable, and shows very little tendency to reversal, even after repeated contact with the allergen. The induction of unresponsiveness depends clearly upon the dose and the time interval between pretreatment and sensitization. 7 days have to elapse after the pretreatment with 500 mg/kg or 3 days after giving 750 mg/kg in order to obtain unresponsiveness to subsequent sensitizing applications. From Chase's experience, it appears that the animals must rest several weeks between the pretreatment (feedings) and the sensitizing procedure. The intravenous procedure has over the feeding procedure the advantage of simplicity, to require only one injection, and to make possible evaluation of dose and time relationships. Regularity and intensity of unresponsiveness seem at least as good as with the feeding method.

When only partial unresponsiveness is achieved, the pretreatment with $DNBSO_3$ impairs to a large extent the reactivity towards DNCB as these animals reach only a low hypersensitivity level.

From Battisto's recently published results (26), it would seem that the injection of the allergen in the mesenteric veins would be especially effective and would require only minute amounts of allergen. A comparative study might be of interest; nevertheless the mesenteric intravenous route is not an absolute requirement.

No anti-DNP antibodies were found by the usual technics in our animals pretreated with $DNBSO_3$ with or without subsequent sensitizing contact with DNCB in spite of the fact that a questionable anaphylactic shock could be induced.

The mechanisms underlying the unresponsive state obtained in our experiments are unknown and can only be theoretically discussed at the present time.

1) The fact that in this system a minimal interval of 3–7 days is required between the pretreatment and the first sensitizing contact could suggest that we are dealing with an "*antibody-induced unresponsiveness*" (22). It could be

assumed that the intravenous injection of $DNBSO_3$ will induce formation of anti-DNP antibodies. The DNP protein conjugates formed upon the subsequent sensitizing epicutaneous application of DNCB would then be readily picked up by already present anti-DNP antibodies and would be destroyed (phagocytosis of antigen-antibody complexes) before having reached the elements responsible for induction of contact-type hypersensitivity. The absence of detectable anti-DNP antibodies and the only questionable anaphylactic reactivity in animals having received one i. v. injection of $DNBSO_3$ make this hypothesis unlikely. The following experiments also speak against this hypothesis:

Animals injected once intravenously with DNP-GPS did *not* show anaphylactic reactivity but became, after sensitizing contact with DNCB, only partially responsive, reaching hypersensitivity levels of 1.5 as compared to controls 2.8 (unpublished data).

Furthermore, animals injected intracutaneously with DNP-BγG or DNP-GPS in Freund's adjuvant, presenting high titers of anti-DNP antibodies and lethal anaphylactic reactivity but practically no contact reactivity to DNCB, became sensitized as normal controls by subsequent epicutaneous application of DNCB (unpublished data).

Sera of Chase's "fed" animals, injected into animals undergoing active sensitization, did not alter the development of dermal sensitization (4). *It is then unlikely that we are dealing here with an "antibody-induced unresponsiveness".*

It cannot be decided if we are concerned with an "immunological paralysis" or "masking" related to the persistence of the antigen, or with an "immunological tolerance" due to a permanent impairment of the capacity to become sensitized.

2) If one considers *immunological paralysis* (15, 16, 17, 18, 23), one could conceive a continuous neutralization of newly synthetized antibodies and/or specific cells by antigen deposits remaining in the tissues. However, direct evidence with radioactive labelled allergen, as well as the failure of tissues of picryl "fed" animals to adsorb anti-picryl antibodies, does not lend support to the "masking" concept (4, 22). Furthermore, one would expect, unless the allergen has no turnover and becomes forever fixed in the tissues, that the neutralization ability would change with time, therefore that both unresponsive and partially responsive animals would become increasingly responsive with time, which is apparently not the case.

3) Finally, the fact that we can obtain totally unresponsive animals which do not respond even to strong and repeated antigenic stimuli suggests that some profound impairment of the sensitizing ability, as conceived for *immunologic tolerance*, (19, 21, 24, 25) may have been achieved. However, it might be argued that the repeated antigenic stimuli are by themselves responsible for the extension of the tolerant state (20). Whether the effect is due to the formation by the allergen of intracellular "immunologic chimara" blocking or preventing antibody formation and/or to the destruction by an overflow of allergen of specifically competent cells according to the clonal hypothesis, cannot be decided (20).

SUMMARY

In adult guinea pigs, an intravenous injection of high doses of 2:4-dinitrobenzene sulfonic acid ($DNBSO_3$) prior to a sensitizing epicutaneous application of 2:4-dinitrochlorobenzene (DNCB) induces a complete or partial but specific state of unresponsiveness to a subsequent contact with DNCB for as long as 38 weeks.

The induction of the unresponsive state seems to be correlated to the dose administered and the time elapsed between the injections of $DNBSO_3$ and the sensitizing application of DNCB.

The incapacity to develop contact type sensitization seems to go along with an impairment of the ability to form circulating anti-DNP antibodies (immunologic tolerance).

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